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Molecular characterization of a novel orthomyxovirus from rainbow and steelhead trout (*Oncorhynchus mykiss*)



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ABSTRACT

A novel virus, rainbow trout orthomyxovirus (RbtOV), was isolated in 1997 and again in 2000 from commercially-reared rainbow trout (*Oncorhynchus mykiss*) in Idaho, USA. The virus grew optimally in the CHSE-214 cell line at 15 °C producing a diffuse cytopathic effect; however, juvenile rainbow trout exposed to cell culture-grown virus showed no mortality or gross pathology. Electron microscopy of preparations from infected cell cultures revealed the presence of typical orthomyxovirus particles. The complete genome of RbtOV is comprised of eight linear segments of single-stranded, negative-sense RNA having highly conserved 5' and 3'-terminal nucleotide sequences. Another virus isolated in 2014 from steelhead trout (also *O. mykiss*) in Wisconsin, USA, and designated SttOV was found to have eight genome segments with high amino acid sequence identities (89–99%) to the corresponding genes of RbtOV, suggesting these new viruses are isolates of the same virus species and may be more widespread than currently realized. The new isolates had the same genome segment order and the closest pairwise amino acid sequence identities of 16–42% with *Infectious salmon anemia virus* (ISAV), the type species and currently only member of the genus *Isavirus* in the family *Orthomyxoviridae*. However, pairwise comparisons of the predicted amino acid sequences of the 10 RbtOV and SttOV proteins with orthologs from representatives of the established orthomyxoviral genera and a phylogenetic analysis using the PB1 protein showed that while RbtOV and SttOV clustered most closely with ISAV, they diverged sufficiently to merit consideration as representatives of a novel genus. A set of PCR primers was designed using conserved regions of the PB1 gene to produce amplicons that may be sequenced for identification of similar fish orthomyxoviruses in the future.

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1. Introduction

The family *Orthomyxoviridae* is currently divided into six genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, *Isavirus* and *Quarantavirus* ([International Committee on the Taxonomy of Viruses, 2014](#)). Among the newer members of the family, the genus *Quarantavirus* was created to include viruses

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detected in ticks and birds from various locations around the world including the Johnston Atoll virus from the central Pacific Ocean and Quarantavirus from Egypt ([Presti et al., 2009](#)). More recently, viruses belonging to a proposed genus, *Influenzavirus D*, have been described from cattle in France and swine and cattle in the United States ([Ducatez et al., 2015](#)). In addition, next-generation sequencing methods are beginning to reveal the presence of many novel viruses in arthropods including 13 quarantaviruses and an unclassified cockroach orthomyxovirus closest to the thogotovirus cluster ([Li et al., 2015](#)). Very recently, an orthomyxovirus-like virus has been described from mass die-offs of tilapia in Israel and Ecuador. Surprisingly, the genome of the tilapia lake virus (TiLV) consisted

of 10 RNA segments with the closest polymerase sequence identity to that of influenza virus C virus (Bacharach et al., 2016).

Orthomyxovirus virions are spherical particles of 80–120 nm that contain surface glycoproteins protruding from a lipid envelope surrounding six to eight linear segments of negative-sense, single-stranded RNA encapsidated by nucleoproteins (International Committee on the Taxonomy of Viruses, 2005). The genome segments range in length from 874 to 2396 nucleotides having partial complementarity in their 5' and 3'-terminal sequences. Typically, two large basic and one acidic polymerase proteins (PB1, PB2, and PA) are encoded and interact to form the RNA-dependent RNA polymerase (RdRp). Orthomyxoviruses also contain a surface glycoprotein (GP) with functions that vary in differing genera to include hemagglutination (HA), esterase activity (HE) and envelope fusion (HEF). Members of the genera *Influenzavirus A* and *Influenzavirus B* have a second envelope glycoprotein, a neuraminidase (NA), which contains sialidase activity (Suzuki et al., 2005) while others may have a fusion protein (F) that lacks sialidase activity (Aspehaug et al., 2005). Two membrane or matrix proteins are encoded from the same RNA segment, the M1 protein is a non-glycosylated matrix protein while M2 functions as a proton-selective ion channel protein (International Committee on the Taxonomy of Viruses, 2005). In addition, another RNA segment typically encodes two non-structural proteins that include NS1 that serves as an antagonist of the type I interferon response and NS2, a putative nuclear export protein (NEP).

Infectious salmon anemia virus (ISAV) is a fish orthomyxovirus that has caused high levels of mortality in farmed Atlantic salmon (*Salmo salar*) in northern Europe, northeastern North America and southern Chile (Falk et al., 1997; Mjaaland et al., 1997; Krossoy et al., 1999; Cottet et al., 2010). To date, all isolates of ISAV belong to a single species within the genus *Isavirus*. This study describes the isolation and characterization of another fish orthomyxovirus, RbtOV, of rainbow and steelhead trout (both *Oncorhynchus mykiss*), that appears to be a member of a novel virus species that, while most like ISAV, is sufficiently distinct to merit consideration as a member of a new genus, proposed name *Mykissvirus*, in the family *Orthomyxoviridae*.

2. Materials and methods

2.1. Source of samples and virus isolation

Two isolates of RbtOV were obtained from samples of early life stage rainbow trout approximately 60 days old at a commercial trout farm in Hagerman Valley, Idaho. The isolates were discovered in 1997 and again in 2000 during health screening of clarified kidney-spleen-liver homogenates when they produced cytopathic effects (CPE) in the CHSE-214 Chinook salmon (*O. tshawytscha*) embryo cell line (Lannan et al., 1984) incubated at 17 °C. Samples were processed following the procedures outlined in the American Fisheries Society Bluebook (USFWS and AFS-FHS, 2014). The isolates were subsequently designated RbtOV-1 and RbtOV-2, respectively.

In 2014, the LaCrosse Fish Health Center (U.S. Fish and Wildlife Service) in Wisconsin isolated another virus, from an ovarian fluid sample of spawning steelhead trout (also *O. mykiss*) collected at a weir in the Kewaunee River near Green Bay, Wisconsin. Samples were processed following the procedures outlined in the American Fisheries Society Bluebook (USFWS and AFS-FHS, 2014) and placed on confluent monolayers of CHSE-214 and EPC (*Epithelioma papulosum cyprini*; *Pimephales promelas*) cell lines (Winton et al., 2010). The cells were observed for 21 days with a blind passage being conducted on day 14. This steelhead orthomyxovirus isolate was subsequently designated SttOV-1.

2.2. Virus challenge of juvenile trout

Unknown replicating agents are occasionally detected during routine operations at fish farms or hatcheries. Shortly after virus isolation, these two unknown replicating agents, RbtOV-1 and RbtOV-2, were injected (50 µL) into groups of juvenile rainbow trout (about 1 g) with fluid from actively replicating CHSE-214 cell cultures or injected with only uninfected cell culture fluid. Fish were monitored for mortality and observed for gross pathology.

2.3. Virus replication kinetics in a fish cell line

In order to test the ability of RbtOV to replicate at various temperatures, monolayer cultures of CHSE-214 cells were prepared in 25 cm² flasks using minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Hyclone) and buffered with sodium bicarbonate. The cultures were incubated overnight at 20 °C. Virus was added to all cell monolayers in the culture flasks except negative control flasks. Individual flasks were incubated at 10, 15, 20, and 25 °C. A small sample (300 µL) of culture fluid was removed from flasks at 5, 8, 11, 14, and 21 days post infection and stored at –80 °C. A plaque assay was developed similar to those for fish rhabdoviruses (Batts and Winton, 1989), whereby preformed monolayer cultures of CHSE-214 cells in 24-well plates were pre-treated with a 7% polyethylene glycol (Sigma-Aldrich, Catalog # P-2263) solution prepared in MEM containing 5% FBS buffered with TRIS (MEM-5-TRIS; pH 7.8). Cells were inoculated with 10-fold virus dilutions, then after 30 min of virus adsorption, a 1 mL volume of overlay (final concentrations of 0.8% methylcellulose (Sigma-Aldrich), MEM, 5% FBS, buffered with TRIS and sodium bicarbonate, pH 8) was applied. For plaque development to occur, infected cells were incubated 12 days at 15 °C then fixed and stained for 1 h with a formalin/crystal violet solution. Viral concentrations are reported as plaque-forming units per milliliter (pfu/ml).

2.4. Electron microscopy

Virus-infected CHSE-214 cells from a 25 cm² flask following 6 days of incubation at 15 °C were removed from the surface using a cell scraper. Following low-speed (250 × g) centrifugation, the cells were placed into a 4% glutaraldehyde solution for 1 h fixation and the fixed cell pellet was prepared for electron microscopy of stained thin sections (Glenn et al., 2012). The supernate was clarified further by centrifugation at 12,000 × g for 30 min at 4 °C using an SW 50.1 rotor (Beckman) and the virus pelleted by additional centrifugation through a 200 µL sucrose (30% w/v) cushion at 77,000 × g for 1.5 h at 4 °C using the SW 50.1 rotor. The virus pellet was resuspended in 50 µL of distilled water and prepared for electron microscopy of negatively stained virions (Hahn et al., 2015). All grids were examined using a JEOL 1400 transmission electron microscope.

2.5. Sanger and next-generation sequencing of the RbtOV genome

Viral RNA was extracted by TriReagent (Sigma-Aldrich) from a 500 µL aliquot of clarified cell culture fluid from a flask of CHSE-214 cells infected with RbtOV following the manufacturer's instructions. Because the virus type was initially unknown, attempts were made at amplifying extracted viral RNA by reverse-transcription polymerase chain reaction (RT-PCR) using in-house sets of DNA primers designed from sequences of known fish viruses (rhabdovirus, paramyxovirus, orthomyxovirus, nidovirus, bunyavirus, picornavirus, hepevirus or aquareovirus), but performing the reverse transcription and annealing at lower stringencies. The PCR conditions used were similar to those for the fish virus, infectious hematopoietic necrosis virus (World Organization for

Animal Health, 2009) that include lower annealing temperatures so that primers do not require exact base pairing. Conditions for RT-PCR were: 30 min reverse transcription at 40 °C followed by 2 min denaturation at 95 °C, then 30 amplification cycles of 95 °C for 30 s, 40 °C for 30 s, and 72 °C for 60 s, and followed by final extension at 72 °C for 7 min. Four primer sets produced amplicon bands and the nidovirus primer set yielded an amplicon with a sequence that BLAST searches showed was similar to the PB1 gene of orthomyxoviruses. Using this authentic sequence, new primers were developed to use in 5' and 3' Rapid Amplification of cDNA Ends (RACE) techniques (Invitrogen) were used according to the manufacturer's instructions to gain sequences directed toward both ends of the PB1 gene segment of RbtOV-1.

In order to obtain additional genomic sequences from additional viral segments, we used cDNA for next-generation sequencing with 36-bp single read Illumina sequencing. The raw reads were aligned with Bowtie (Langmead et al., 2009) and non-viral sequences (e.g. mycoplasma, ribosomal RNA, etc.) were discarded from analysis. Remaining sequences were assembled *de novo* with the VELVET assembler (Zerbino and Birney, 2008) with a minimum contig length set to 30 nt. To identify putative RbtOV segments, resulting contigs were aligned to the protein sequences of ISAV using BLASTx. Contigs that had a significant alignment score were aligned to the protein sequences using CLUSTAL to verify the validity of the annotation of the four largest viral segments. Primer sets were designed from conserved regions of ISAV and influenzaviruses to allow amplifying portions of the other genome segments, and finally each segment was completed with 5' and 3' RACE methods to obtain all 16 segment termini. Sequence data were edited using SEQUENCHER 4.1 (Gene Codes Corporation) and aligned with the CLUSTALW algorithm in MACVECTOR 6.0 software (Accelrys). Using the methods above, a partial sequence of the PB1 gene was also obtained for the other rainbow trout virus isolated in 2000, known as RbtOV-2.

2.6. Next generation sequencing of the SttOV-1 genome

Since the isolate from steelhead trout produced a type of cytopathic effect in cell culture that did not appear typical of known fish viruses, it was sent to a laboratory for next-generation sequencing. To facilitate the characterization of viral genomes, a bioinformatics pipeline consisting of *de novo* assembly and BLAST was employed. First, Cutadapt (Martin, 2011) was used to trim adapter sequence and filter out reads below Phred quality scores of 20. Sequence reads that passed quality filter were then assembled into contigs using SPAdes (Bankevich et al., 2012). The contigs were then screened for virus sequences using BLASTn and BLASTx searches against GenBank non-redundant nucleotide and protein databases (Camacho et al., 2009). Finally, full genomes or contigs were inspected using Geneious (Biomatters, Auckland, New Zealand).

2.7. Sequence comparisons

Both nucleotide and deduced amino acid sequences for each segment of RbtOV were analyzed by pairwise comparison to their orthologs of the Glesvaer strain of ISAV (GenBank Numbers: ADR77505-ADR77514) and to representative type species for other genera of the family *Orthomyxoviridae*. These included Influenzavirus A/PR/8/34 (GenBank ACF41841, ACF41842, ACF41844, NP_040980 to NP_040982, AGU93024, P03508, ABD77676, AAM75162) and Influenzavirus B/Lee/40 (GenBank NP_056657 to NP_056666, YP_419283), members of the two other orthomyxovirus genera with eight genomic segments, as well as Influenzavirus C/Ann Arbor/1/50 (GenBank YP_089652 to YP_089658, YP_002302328, YP_002302329) and Thogotovirus

strain Si Ar 126 (YP_145794, YP_145795, YP_145805, YP_145806, YP145808 to YP145810). When BLAST alignments showed low levels of sequence identity, attempts were made to align RbtOV segment sequences with homologous proteins of the type species of each orthomyxovirus genus. Analyses included calculations of the total nucleotide length, number of amino acids in the open reading frame, calculated pI values and number of potential N-linked glycosylation sites for the various proteins of RbtOV and their comparison with analogous data from the representative type species for the other genera. Identities were also compared between the available amino acid sequences of the corresponding segments of the RbtOV-1 and SttOV-1 strains of the virus, as well as for the partial PB1 sequence of the RbtOV-2 strain.

To compare all of the proteins of RbtOV and SttOV with their orthologs from other genera of the family *Orthomyxoviridae*, pairwise protein identities of sequences from up to 10 orthomyxoviral proteins of selected representative isolates were calculated and displayed using a species demarcation tool with previously described parameters (Ng et al., 2015; Muhire et al., 2014).

2.8. Phylogenetic analyses

In order to compare RbtOV and SttOV with other orthomyxoviruses, two representatives of each established genus were selected for phylogenetic analysis of their PB1 amino acid sequences as follows: ISAV-Glesvaer/2/1990 (ISAV_Nor; ADR77506), ISAV-Canada/CCBB (ISAV_Can; AAL67962), Influenzavirus A/PR/8/1934 H1N1 (FluA34; ABO21706), Influenzavirus A/swine/Texas/4199-2/1998 H3N2 (FluA98; AEK70339), Influenzavirus B/Lee/1940 (FluB40; AAA43767), Influenzavirus B/Yamagata/16/1988 (FluB88; ABL77264), Influenzavirus C/Ann Arbor/1/1950 (FluC50; BAD24938), Influenzavirus C/Johannesburg/1/1966 (FluC66; AAF89738), Influenzavirus D/swine/Oklahoma/1334/2011 (FluD11; AFJ19019), Influenzavirus D/bovine/France/2986/2012 (FluD12; CEE50061), Quarantil/1953 (QRFV53; ACY56282), Johnston Atoll/1964 (JAV64; ACY56284), Thogotovirus (Thogoto; YP_145794), Dhori virus/1313/61 (Dhori; ADF56030), RbtOV-1/1997 (RbtOV1; KX882062), and SttOV-1/2014 (SttOV1; KX882070). Nucleotide or inferred amino acid sequence data were aligned in CLUSTALX (Qt/QMake Software), and manually inspected for anomalies. Maximum likelihood phylogenetic analysis was performed with RAxML v8.1.22 (Stamatakis, 2015) using the rapid tree search algorithm and the general time-reversible substitution model for nucleotide sequence data and the Blosum62 model for amino acid sequence data (Henikoff and Henikoff, 1992). Phylogenetic analysis using Bayesian MCMC methods was performed using the BEAST software package v1.8.0 (Drummond and Rambaut, 2007). For all Bayesian analyses, a strict clock prior and the speciation birth-death process with incomplete sampling tree prior was used (Stadler, 2009). The substitution models were the HKY model for nucleotide sequence data (Hasegawa et al., 1985) and the Blosum62 model for amino acid sequence data (Henikoff and Henikoff, 1992). The phylogenetic tree is derived from three separate MCMC analyses of 150 million generations that each achieved convergence and good mixing, and were subsequently combined, summarized, and analyzed. Results were analyzed, annotated, and drawn using the complementary suite of programs in BEAST, including Tracer v1.6 and FigTree v1.4.2.

2.9. RT-PCR for detection of fish orthomyxoviruses

Nucleotide sequences of highly conserved regions in the PB1 polymerase gene of RbtOV-1 and a strain of ISAV from Canada (NBISA01; GenBank Accession DQ520595) were used to select primer sites for development of RT-PCR assays that might be used

detect various fish orthomyxoviruses in North America. Three conserved primer sites were identified that allowed development of a semi-nested RT-PCR to detect very low levels of viral RNA using degenerate primer sets. The first round RT-PCR primers, 5'-YTN ACW GGA GAY AAC TCN AA-3' and 5'-GTM ACR AAG TCR TCT GAR CTY TG-3' were designed to produce a 410 bp amplicon and the semi-nested PCR primers, 5'-YTN ACW GGA GAY AAC TCN AA-3' and 5'-TTY GCC ATW CCC ATY ARC ATY CC-3', would produce a 317 bp amplicon. The PCR conditions used were those for the fish virus, infectious hematopoietic necrosis virus (World Organization for Animal Health, 2009) that include: 30 min reverse transcription at 50 °C followed by 2 min denaturation at 95 °C, then 30 amplification cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, and followed by final extension at 72 °C for 7 min.

3. Results and discussion

3.1. Source of samples and virus isolation

The RbtOV-1 and RbtOV-2 isolates from juvenile rainbow trout produced initial CPE beginning approximately 7 days after inoculation of CHSE-214 cell cultures incubated at 17 °C. At one week post-inoculation, discreet clusters of small round refractile cells were observed similar to that shown for SttOV-1 in Fig. 1. Over the next two weeks the CPE progressed throughout the entire cell monolayer with 50–60% of the cells exhibiting this characteristic. The CPE was definitive, but the infection seemed somewhat self-limiting as it did not progress to involve the entire monolayer.

For the SttOV-1 isolate, the virus was not detected during the initial 21-day observation period of CHSE-214 cells at 15 °C; however, a blind-passage of these ovarian fluid samples obtained from spawning steelhead trout produced CPE 16 days later. The SttOV-1 isolate produced CPE similar to that of the RbtOV-1 and RbtOV-2 viruses as the cells rounded, slowly spreading throughout the monolayers (Fig. 1). Samples inoculated on EPC cells failed to develop CPE. Subsequent testing of juvenile offspring from the same steelhead population did not produce viral CPE.

The prevalence of these fish orthomyxoviruses in trout populations appears to be relatively low as the RbtOV-1 or RbtOV-2 isolates were not found again during several decades of intensive fish health surveillance activities conducted by the commercial trout farm in Idaho, and the SttOV-1 isolate was only found on a single occasion from fish examined from approximately 3300 steelhead from Ohio, Illinois and Wisconsin since 2006. No additional samples from the Keweenaw River site have been positive for this virus collected during 2013–2016. However, it is likely that rainbow or steelhead trout are not the only hosts for these viruses and additional work is needed to better define their host and geographic range.

3.2. Virus challenge of juvenile trout

Challenge studies of juvenile rainbow trout showed no mortality or gross pathology associated with exposure to this virus. Future studies should also test the susceptibility of other salmonid and non-salmonid species to determine if these viruses represent significant risks to populations of farmed or wild fish.

3.3. Virus replication kinetics in a fish cell line

A viral plaque assay was developed to determine the growth kinetics of the RbtOV-1 isolate. Optimal replication of the RbtOV-1 isolate occurred at 15 °C in the CHSE-214 cell line, reaching a virus concentration of $10^{6.4}$ pfu/ml on day 11. Maximal virus concentration at 10 °C was approximately the same, $10^{6.3}$ pfu/ml; however, 14 days of incubation were required to reach this level. Infected

cells at 20 °C replicated the virus faster, but to a much lower titer of $10^{5.0}$ pfu/ml on day 8. No virus was detected from any samples removed from flasks incubated at 25 °C, signifying that the infectious virus was below the $10^{1.4}$ pfu/ml detection limit of the assay. No efforts were made to test the susceptibility of other cell lines to either the RbtOV-1 or SttOV-1 isolates including fish cell lines commonly used to support the growth of ISAV (e.g. SHK-1, ASK-2). However, these fish orthomyxoviruses are somewhat similar to North American strains of ISAV that also replicate in the CHSE-214 cell line (Bouchard et al., 1999; Kibenge et al., 2000).

3.4. Electron microscopy

Electron microscopy of negatively stained grids revealed roughly spherical virions of approximately 100–120 nm that had morphological features typical of orthomyxoviruses (Fig. 2A). Thin sections showed particles of a similar size budding from cell membranes (Fig. 2B). No particles characteristic of other vertebrate viruses were observed in the preparations.

3.5. Amplification and sequencing of the RbtOV genome

The entire genome of the RbtOV-1 isolate from rainbow trout was determined using a strategy based on RT-PCR amplification of RNA from clarified cell culture fluid and next-generation sequencing to obtain authentic genome segment portions. Next-generation sequencing of the uncharacterized RbtOV cDNA generated 3.4 million reads. About 64% of these were non-viral (primarily mycoplasma origin) and were discarded. The remaining 1.2 million reads were assembled into 5056 contigs for which the average size was 121 nt. Significant BLASTx hits against ISAV protein sequences were obtained for 20 contigs, characterizing large portions of the segments 1–4 of RbtOV. No significant alignments were obtained for the remaining segments. However, portions of segments 5 through 8 could be determined by sequencing RT-PCR amplicons generated using gene-specific primer sets made from conserved regions of other orthomyxovirus segments. All eight segments of the RbtOV-1 isolate were completely sequenced by traditional Sanger sequencing methods and 5' RACE techniques were used to obtain the exact terminal sequences. The complete genome of the RbtOV-1 isolate was 13,530 nt in length, similar to the 13,227 nt of the ISAV genome and the 13,588 nt for that of Influenzavirus A (FLUAV).

Properties of the predicted proteins encoded by the eight segments of RbtOV are summarized in Table 1. The size order of the eight segments, based on total nucleotide length, was as follows: PB2, PB1, NP, PA, NA, HA, NS1/NEP, and M1/M2. This size order is similar to that of ISAV. However, compared to FLUAV and influenza B virus (FLUBV), ISAV has a fusion protein (F) instead of a neuraminidase and the hemagglutinin has esterase activity (HE), features which have not been ascertained for RbtOV (Table 2). The segment encoding the NP of isolate RbtOV and ISAV was larger than the segment encoding the PA, which is not the case for FLUAV or FLUBV. Also, the sizes of RbtOV segments 7 and 8 were reversed with respect to their orthologs in FLUAV or FLUBV (Table 2).

A partial sequence of 1228 nt was obtained for the PB1 gene of the RbtOV-2 isolate from rainbow trout isolated in 2000 for comparison to the 1997 isolate, RbtOV-1. Alignment showed identities of 99.8% and 99.9% at the nucleotide and amino acid levels, respectively, indicating the isolates are the same virus. For the 2014 isolate from steelhead trout in the Great Lakes, SttOV-1, next generation sequencing methods rapidly produced a nearly complete genome lacking only the terminal nucleotide sequences. Alignment of the deduced amino acid sequences for the genome segments of SttOV-1 with those of RbtOV-1 showed high levels of identity (Fig. 3). These amino acid identities ranged from 89% for the HA (segment

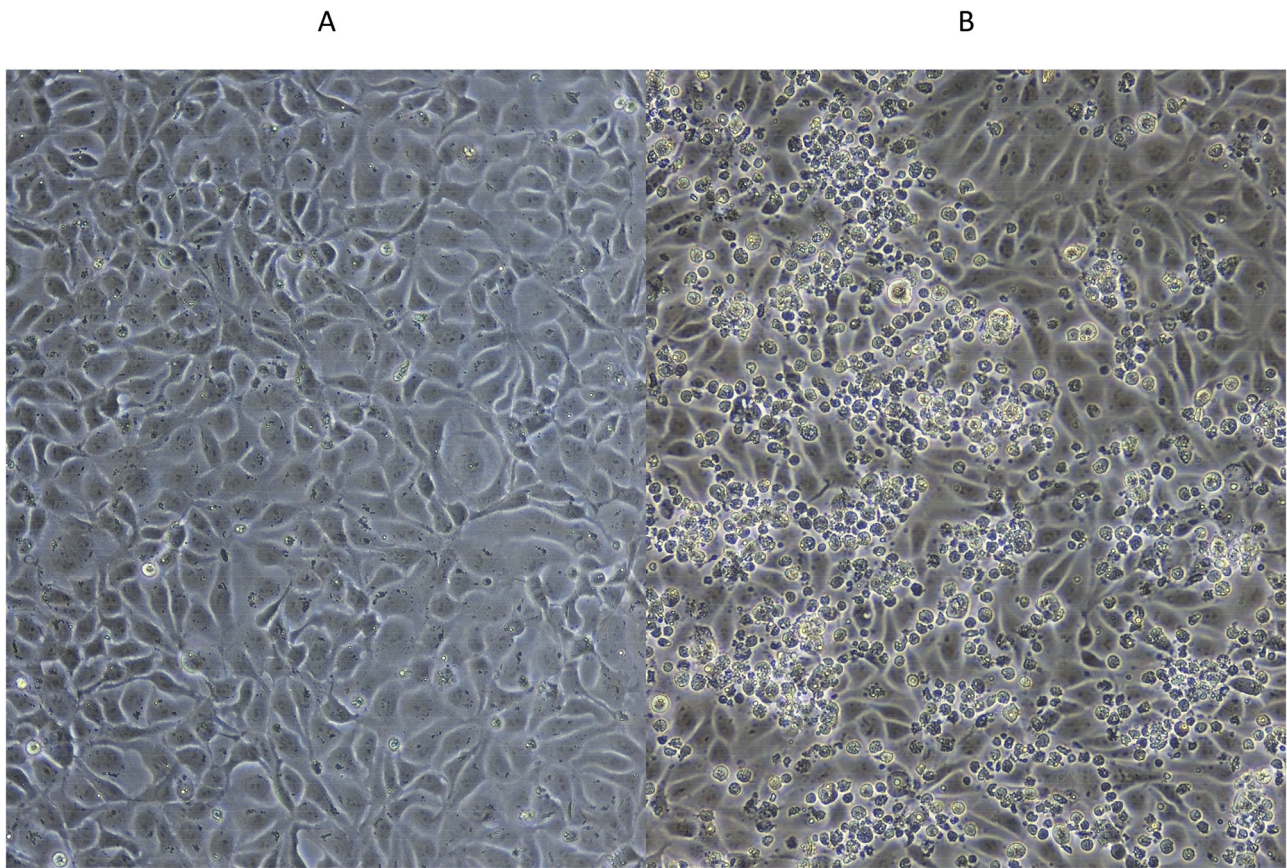


Fig. 1. Cytopathic effect, at 20 \times magnification, produced by SttOV-1 in monolayers of Chinook salmon embryo cells (CHSE-214) incubated at 15 $^{\circ}$ C. (A) Normal cells. (B) Infected cells.

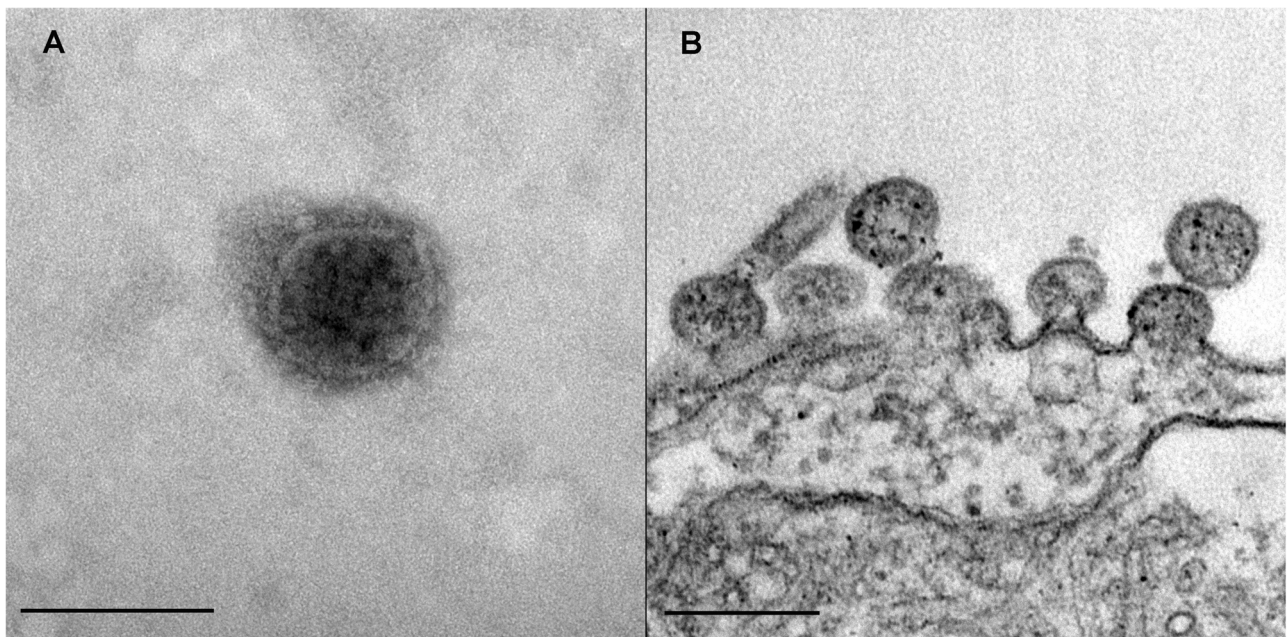


Fig. 2. (A) Virion stained using 1% phosphotungstic acid and (B) cells stained using 2% uranyl acetate and 0.4% lead citrate. Scale bars = 200 nm. Images were processed with Adobe Photoshop and included application of the unsharp mask filter in Image 2B.

6) to 99% for the other segments of the SttOV-1 isolate. Relative to RbtOV-1, there was a single amino acid insertion in the HA of SttOV-1 (following aa 433) where a string of G nucleotides encodes an extra glycine residue.

The complete nucleotide sequences of the RbtOV-1 isolate have been deposited in GenBank under accession numbers KX882061 to KX882068. The partial PB1 sequence of isolate RbtOV-2 is

Table 1

Properties of the genome segments of rainbow trout orthomyxovirus (RbtOV). Abbreviations are defined as follows: FLUAV = influenza virus A, ISAV = infectious salmon anemia virus, UTR = untranslated region, Orf = open reading frame, nt = nucleotide, aa = amino acid, kDa = kiloDaltons, pI = calculated isoelectric point.

RbtOV segment	FLUAV/ISAV ortholog	Segment length (nt)	5' UTR length (nt)	Orf length (aa)	3' UTR length (nt)	Predicted protein (kDa)	Calculated pI
1	PB2	2256	8	727	67	80.6	9.0
2	PB1	2187	27	701	57	79.3	6.9
3	NP	2081	48	638	119	70.1	8.7
4	PA	1875	34	588	77	67.4	8.7
5	NA (F)	1605	44	480	121	53.8	6.0
6	HA (HE)	1495	8	464	95	51.3	5.1
7	NS1/NEP	1097	42/580	306/143	137/88	34.3/16.2	8.8/4.5
8	M1/M2	934	37/87	207/235	276/142	23.2/25.6	9.4/9.4

Table 2

Comparison of genome segments of RbtOV with those of selected orthomyxoviruses containing eight genomic segments. Segments are ranked in order of size. For each virus segment the encoded protein abbreviation and nucleotide length are shown. RbtOV strain is RbtOV-1, ISAV strain is Glesvaer/2/90, FLUAV strain is Influenzavirus A/PR/8/34 (H1N1), and FLUBV strain is Influenzavirus B/Lee/40. Protein abbreviations are: PB2 = basic polymerase protein 2, PB1 = basic polymerase protein 1, NP = nucleocapsid protein, PA = acidic polymerase protein, NA-F = neuraminidase or fusion protein, HA-HE = hemagglutinin or hemagglutinin esterase protein, NS1 = nonstructural protein 1, NS2 = nonstructural protein 2, NEP = nuclear export protein, M1 = matrix protein 1, and M2 = matrix protein 2.

Ranked size	RbtOV	ISAV	FLUAV	FLUBV
1	PB2 2256	PB2 2267	PB2 2341	PB2 2396
2	PB1 2187	PB1 2249	PB1 2341	PB1 2369
3	NP 2081	NP 2046	PA 2233	PA 2308
4	PA 1875	PA 1826	HA 1778	HA 1882
5	NA 1605	F 1485	NP 1565	NP 1841
6	HA 1495	HE 1321	NA 1413	NA 1557
7	NS1/NEP 1097	NS1/NEP 1127	M1/M2 1027	M1/M2 1191
8	M1/M2 934	M1/M2 906	NS1/NEP 890	NS1/NS2 1096

KX880091, and SttOV-1 segment sequences are KX882069 to KX882076.

3.6. Complementarity of the 5' and 3' termini

Each genome segment of the RbtOV-1 isolate showed a high degree of inverse complementarity of the 5' and 3' termini as well as a high degree of conservation between the corresponding terminal sequences of the various segments (Fig. 4). Nearly complete complementarity was present for the seven terminal nucleotides (UCGUUUC) of each RbtOV-1 segment, while good complementarity was observed for up to 24 nucleotides from each genomic terminus. The consensus sequence for each terminus included 16 of 24 identical nucleotides (Fig. 4). Two of the segments, encoding PB2 and HA, have the first methionine of the AUG protein translation start site within the region of complementarity beginning at nucleotide position 9. However, both sites are in adequate context for translation initiation at positions -2 and -3 (Kozak, 1991) and both maintain similar reverse complementarity with the other termini even though coding for amino acids. Interestingly, ISAV also has very short 3' (genome sense) untranslated regions of 7–8 nt at the start of genes encoding the PB2, HE, and F proteins (Merour et al., 2011). The 5' UTRs (genome sense) of each segment of RbtOV were 57–142 nt in length (Table 1), more similar to ISAV at 67–147 nt and longer than to FLUAV at only 20–58 nt (Merour et al., 2011). Upstream of the polyadenylation signal was another conserved region in at least 7 of the 8 segments having nucleotide sequence “GUKURAUUGYAMAU”. This conserved domain was in the same location as the “GNNNUUGANNNU” domain described for ISAV by Merour et al. (2011).

3.7. Polymerase proteins

Among orthomyxoviruses, three segments encode relatively conserved proteins that interact to form the RdRp including two basic (PB1 and PB2) and one acidic (PA) polymerase proteins. Segment 1 of the RbtOV-1 isolate encodes an ortholog of PB2, segment

2 encodes an ortholog of PB1, and segment 4 encodes an ortholog of PA. Surprisingly, the PA protein of RbtOV-1 was quite basic with a predicted pI of 8.6, while PB1 was much less basic than expected with a predicted pI of 6.9 (Table 3). The PB2 protein was highly basic as expected having a predicted pI of 9.0. Therefore, while the RdRp of RbtOV appears to be composed of one slightly acidic and two basic proteins, they are not the same as found in ISAV or the influenzaviruses. For example, the PB2, PB1, and PA proteins of ISAV have pIs of 8.4, 9.3 and 7.1, respectively, and those of Thogotovirus (THOGV) and Influenza A or B viruses have even more pronounced differences (Table 3).

The PB2 gene transcription unit on segment 1 contains a single large orf encoding a 727-amino acid protein with a calculated mass of 81 kDa (Table 1). The first AUG begins very early with only eight untranslated nucleotides upstream, but is in strong context for translation initiation (Kozak, 1991). The next AUG is in a less optimal context at about 150 nt downstream, suggesting that the first AUG is the most likely start site. The PB1 gene transcription unit on segment 2 contains a single large orf encoding a 701-aa protein with a calculated mass of 79 kDa (Table 1). For PB1, the first AUG occurs at nt 28 and is in strong context for translation initiation. The PA gene transcription unit on segment 4 contains a single large orf encoding a 588-aa protein with a calculated mass of 67 kDa. The PA of RbtOV is similar in size to that of ISAV at 578 aa, but much smaller than the 709–726 aa PA of influenzaviruses (Table 3). The first AUG of the sequence of the PA of RbtOV is in strong context for translation initiation.

Pairwise alignments of the amino acid sequences of the three polymerase proteins of RbtOV with orthologs of other orthomyxoviruses showed the highest identities with ISAV for all three proteins (Fig. 3). As expected, the most conserved polymerase protein, PB1 was the highest in percentage amino acid identity of 42% with that of the Glesvaer strain of ISAV (ADR77506) and ISAV-Canada/CCBB (AAL67962), revealing similar identities using sequences from either strain of ISAV. Therefore, we selected the Glesvaer strain of ISAV for all additional comparisons. The ISAV PA had 33% and ISAV PB2 had 31% amino acid identity with RbtOV. The

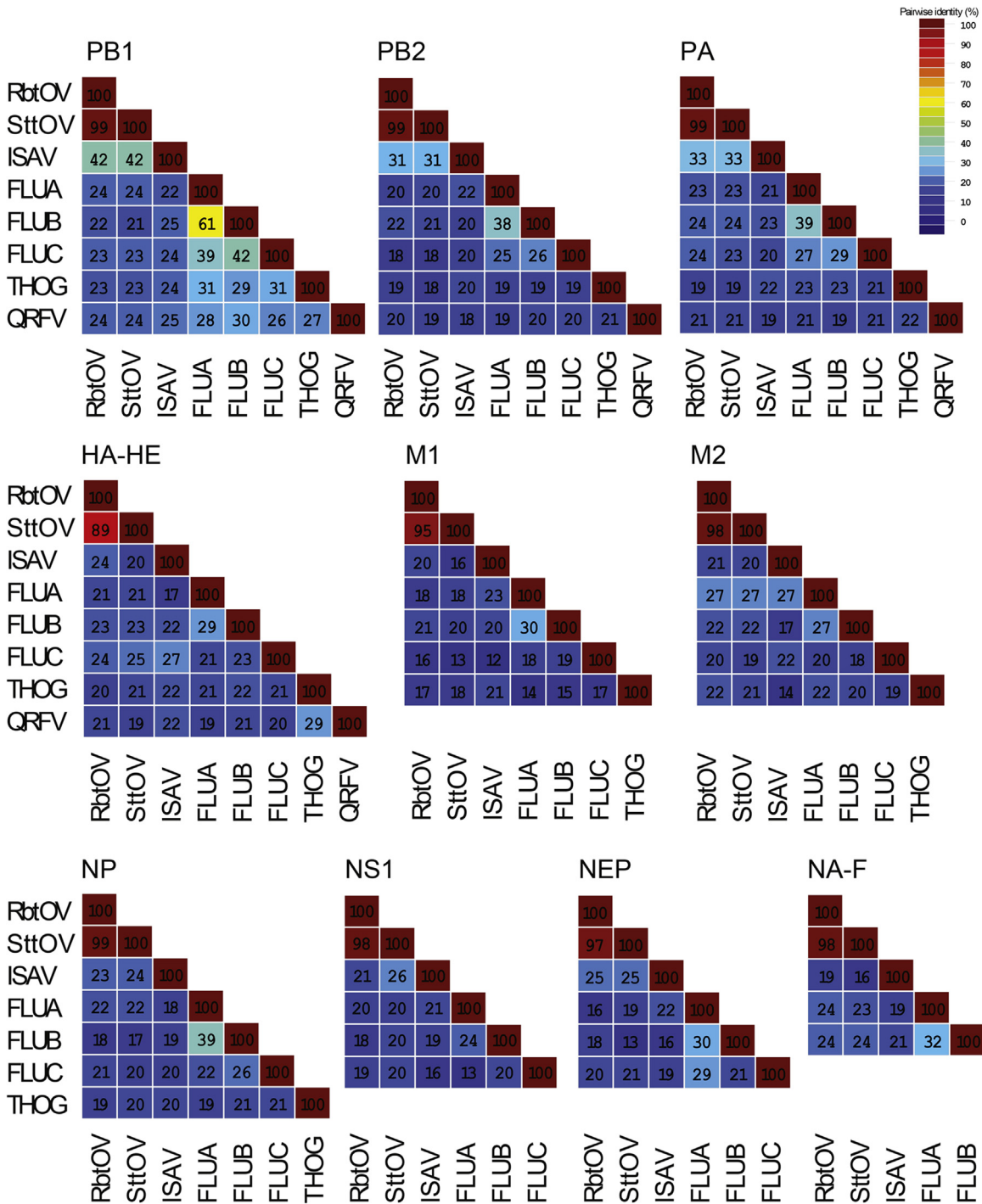


Fig. 3. Pairwise comparisons of representative orthomyxoviruses of different genera in the PB1, PB2, PA, HA-HE, M1, M2, NP, NS1, NEP, and NA-F proteins. Each number represents the pairwise amino acid identities between the corresponding species. RbtOV = rainbow trout orthomyxovirus, SttOV = steelhead trout orthomyxovirus, ISAV = infectious salmon anemia virus, FLUA = influenza virus A, FLUB = influenza virus B, FLUC = influenza virus C, THOG = thogotovirus, and QRFV = Quaranfil virus.

amino acid identity with other orthomyxovirus polymerase proteins was quite low, ranging from 20 to 24% for FLUA, 22–24% for FLUB, 18–23% for influenza C virus (FLUC), 19–23% for THOG, and 20–24% for QRFV.

Alignment of the PB1 sequence of RbtOV with that of ISAV revealed several blocks of amino acids where as many as 15 consecutive residues such as “EMALKVSKAAGLNVS” beginning at nt 1384 and “LTGDNSK” beginning at nt 928 of the RbtOV-1 sequence were identical. The latter region was similar to the FLUA signature sequence of “ITGDNTK”, a conserved “GDN” polymerase motif that is also found throughout the order *Mononegavirales* (Poch

et al., 1990). In addition, the PB1 proteins of both RbtOV and ISAV-Glesvaer contain all four conserved RdRp motifs (I–IV) analyzed by Chu et al. (2012) and shown in Fig. 5. The short motif I was completely conserved among FLUA, RbtOV and ISAV while the other motifs were 46–73% identical to the amino acid sequence of FLUA.

3.8. Nucleoprotein

The nucleoprotein (NP) of orthomyxoviruses is a group-specific protein that is associated with each genomic ssRNA segment in the form of a ribonucleoprotein complex. Contrary to that of most

RbtOV-1 segment	3' terminus (leader)	5' terminus (trailer, reverse complement)
1 (PB2)	3'-UCG UUU <u>CCU ACC</u> UGA AGG AUU GGU 17	5'-UCG UUU CUU UCC UGA AAA UAA GUA 19
2 (PB1)	UCG UUU CUU GCC AAG UUA AAU UUU 20	UCG UUU CCU UUC CAA GAA AAA GUG 18
3 (NP)	UCG UUU CCU UCA GGA AUU AAU GUU 20	UCG UUU CUU GCA GGA AAA AAU UAG 22
4 (PA)	UCG UUC CUU AGC AAG AAU UAA UUU 18	UCG UUU CUG AGC AAG AAA AAA UAA 16
5 (NA)	UCG UUU CUG ACA GAG AUU AAU UAA 21	UCG UUU CUU GCA GAG AAA AAU AUG 21
6 (HA)	UCG UUU CUU <u>ACA</u> GAG GUU AUA ACA 19	UCG UUU CCA UCA GAG AAA AAU GUG 21
7 (NS1/NEP)	UCG UUU CCU AGG AGA AUU UUU GUG 16	UCG UUU CUG AGG AGA AAA UUU AGU 15
8 (M1/M2)	UCG UUU CUU AAG GAG AUU UUA ACA 17	UCG UUU CCA AAA GGA GAA AAU UAU 18
Consensus	UCG UUU CUU ACM GAG AUU AWU KUU	UCG UUU CUU WCA GRA AAA AAU KUG

ISAV segment	3' terminus (leader)	5' terminus (trailer, reverse complement)
1 (PB2)	3'-UCG AUU <u>CUU ACC</u> UGA AAU AUA GUC 18	5'-UCA UUU UUU ACC UGU AAA AUA ACU 19
2 (PB1)	UCG UUU <u>CUU GCG</u> AGA AAU UAU UGG 16	UCA UUU UUU ACG AGA AAA UGA AUA 21
3 (NP)	UCG UUU CUA ACG AGU UUA GGG UUU 16	UCA AUU UUA ACG AGA AAA GAA AUA 21
4 (PA)	UCG AUU CUA ACC GAC AAA GUU <u>CUA</u> 20	UCA UUU UUA ACC GAA AAA CCU UUU 19
5 (F)	UCA AUU <u>UCU ACC</u> GAA AAG AUU GUU 15	UCA UUU UUA ACC GAU AAA UAU GUU 20
6 (HE)	UCG UUU <u>CUA CCG</u> UGC UAA GUA UUA 17	UCA UUU UUU ACG UGA AAA GAC AUU 21
7 (NS1/NEP)	UCG AUU CUA <u>AGA</u> GGA AUA UGU <u>UAC</u> 19	UCA UUU UUA AGA GGA AAA GCA AAA 20
8 (M1/M2)	UCG UUU CUA ACC GAU AGA UGG <u>UAC</u> 18	UCA UUU UUU ACC GAA AAA UAG AAA 21
Consensus	UCG WUU CUA ACC GGA AAA KUU UUC	UCA UUU UUU ACC GGA AAA KAA AUW

Fig. 4. RbtOV and ISAV genome segments with each 3' leader and 5' trailer terminal regions shown as genomic sense RNA. The 5' terminus is in reverse complement orientation for comparison. Underlined nucleotides UAC indicate the site of the first AUG methionine codon for protein translation. In the consensus sequences, M, W, K, R refer to variable sites according to the convention used by the International Union of Pure and Applied Chemistry. Bold letters indicate nucleotides that do not match with the consensus sequence. Numbers following sequence are nucleotide matches with the consensus 24 nucleotides.

Table 3

Comparisons of representative orthomyxovirus proteins. Within each box are: segment number arranged by total nucleotide length, number of amino acids in the open reading frame and calculated pI values. Within parentheses is the number of potential N-linked glycosylation sites, determined by having amino acid sequences N-X-S/T-X, where X is not a proline. na = not available.

Protein abbreviation	RbtOV-1 345/97	ISAV Glesvaer/2/90	Influenza A/PR/8/34	Influenza B/Lee/40	Influenza C/Ann Arbor/1/50	Thogoto Si Ar 126
PB2	1 727aa 9.0	1 722aa 8.4	1 759aa 9.6	1 770aa 9.4	1 774aa 9.3	1 769aa 9.0
PB1	2 701aa 6.9	2 708aa 9.3	2 757aa 9.5	2 752aa 9.1	2 754aa 9.0	2 710aa 7.9
NP	3 638aa 8.7	3 616aa 8.3	5 498aa 9.5	5 560aa 9.4	5 565aa 9.6	5 454aa 9.4
PA	4 588aa 8.6	4 578aa 7.1	3 716aa 5.2	3 726aa 5.4	3 709aa 7.8	3 622aa 5.7
NA-F	5 480aa 6.0 (4)	5 F 444aa 7.4 (2)	6 455aa 8.1 (5)	6 466aa 7.0 (4)	na	na
HA-HE	6 464aa 5.1 (6)	6 HE 391aa 8.6 (2)	4 566aa 7.5 (7)	4 584aa 8.0 (10)	4 HEF 655aa 5.9 (8)	4 G75 512aa 5.8 (5)
NS1	7 306aa 8.8 (1)	7 300aa 5.9 (1)	8 230aa 6.2 (1)	8 281aa 5.9 (5)	7 246aa 9.2 (1)	na
NEP	7 143aa 4.4 (1)	7 159aa 4.4 (1)	8 121aa 5.0 (0)	8 122aa 6.4 (2)	7 182aa 8.1 (2)	na
M1	8 207aa 9.4 (3)	8 196aa 9.2 (1)	7 252aa 9.7 (0)	7 M 248aa 9.3 (1)	6 M 242aa 9.3 (2)	6 M 266aa 6.6 (1)
M2	8 235aa 9.4 (1)	8 241aa 5.6 (2)	7 97aa 5.6 (1)	7 BM2 109aa 5.7 (0)	6 CM2 139aa 4.4 (1)	6 ML 304aa 5.2 (1)

orthomyxoviruses, the nucleoprotein of RbtOV and ISAV is encoded from segment 3, and not from segment 5 as in the influenzaviruses (Tables 1, 2). The larger genomic segment encoding the NP of RbtOV corresponds to a larger orf of 638 aa having a calculated mass of 70.1 kDa, similar to the NP of ISAV at 616 aa (Table 3). In contrast, the NP of other orthomyxoviruses ranges in size from 454 to 565 aa. The first AUG codon of the RbtOV orf encoding the NP is in strong context for translation initiation. Surprisingly, the NP of RbtOV had a calculated pI of 8.7, although long stretches of acidic residues were observed such as "EEEEEEAQEE" beginning at nt 296. The pI for the NP of RbtOV was similar to that of ISAV at 8.3, but less basic than the other orthomyxoviruses that range from 9.4–9.6 (Table 3). Pair-

wise alignments indicated that the NP of RbtOV had relatively low amino acid identity with orthologs in other members of the family *Orthomyxoviridae*, ranging from 19% for Thogotovirus, 18–22% for influenzaviruses, and 23% for ISAV (Fig. 3).

3.9. Neuraminidase/fusion protein

An envelope glycoprotein predicted to be the putative neuraminidase (NA) of RbtOV is encoded on segment 5, although for influenzaviruses A and B the NA is encoded by segment 6. For ISAV, an ortholog of the NA has been characterized and is also located on the fifth largest segment, although it is not known to have sial-

<u>Motif I</u>	FLUAV	TGDN
	RbtOV
	ISAV
<u>Motif II</u>	FLUAV	LSPGMMMGMF
	RbtOV	APR..L...A
	ISAV	VRR..L...A
<u>Motif III</u>	FLUAV	WDGLQSSDDFALI
	RbtOV	VLTK.....VTC
	ISAV	VYT.....VTG
<u>Motif IV</u>	FLUAV	GINMSKKKSYI
	RbtOV	.L.V.P.....
	ISAV	.L.V.Q...FY

Fig. 5. Conserved motifs in PB1 proteins of Influenzavirus A/PR/8/34 (H1N1) aligned with PB1 amino acid sequences of RbtOV-1 and ISAV-Glesvaer. Locations of amino acid motifs on Influenzavirus A are: I aa 303–306, II aa 403–412, III aa 438–450, and IV aa 474–484. Dots indicate amino acid identity with FLUAV.

idase activity and is thus termed a fusion (F) protein (Aspehaug et al., 2005). The orf encoding the NA of RbtOV begins with an AUG in strong initiation context, producing a predicted protein of 480 aa. The predicted amino acid sequence of the neuraminidase of RbtOV produced alignments having a 23–24% identity with the NA of influenza virus A or B (Fig. 3). The three viruses shared nine conserved cysteine residues in the carboxyl half of the protein, reminiscent of a zinc finger domain present among the V proteins of several paramyxoviruses (Lamb and Kolakofsky, 2001). Surprisingly, the RbtOV neuraminidase sequence showed no significant similarity to any ISAV protein, although manually, a 16% amino acid identity could be obtained by pairwise comparison with that of the F protein of ISAV. An unusually high number of threonine residues were predicted to be encoded in the RbtOV neuraminidase, 14%, compared to the other proteins of the virus where threonine content ranged from only 4–8%. This was especially evident for aa 52–89 of the NA, where 21 threonine residues out of 38 residues (55%) were located. As shown in Table 3, the neuraminidase of RbtOV was somewhat acidic (pI 6.0) compared to the F protein of ISAV or the NA of influenza viruses A and B (pI 7.0–8.1). While the sialidase activity of RbtOV was not tested, BLAST searches using the full amino acid sequence of the RbtOV neuraminidase indicated it was most like the NA of the N3 strains of influenza virus A and related to the sialidase superfamily of proteins having sialidase propeller motifs 2–6. Four N-linked glycosylation sites were identified in the amino acid sequence of the RbtOV neuraminidase, similar to the four or five sites found in the NA of influenza virus A or B and greater than the two present in the NA of ISAV (Table 3).

3.10. Hemagglutinin protein

A second envelope glycoprotein, the hemagglutinin (HA), is encoded by segment 6 of RbtOV. For members of the family *Orthomyxoviridae*, the function of this protein, whether involved in hemagglutination, neutralization, envelope fusion, or esterase activity, varies with the genus. The HA gene of RbtOV contains an orf beginning with an AUG in adequate initiation context only nine nucleotides from the leader terminus and within the region of complementarity, a feature similar to that of ISAV (Merour et al., 2011). Initiation at this start site is predicted to encode a protein of 464 aa (Table 1). The next strong, in-frame AUG codon is at nt 156 and may serve as an alternate start for protein synthesis. The predicted amino acid sequence of the RbtOV hemagglutinin did not produce a significant match with any orthomyxovirus sequence in the NCBI

database when searched using BLASTp. Instead, the percent amino acid identities ranged between 20 and 24% for all of the representative orthomyxoviruses compared (Fig. 3), including ISAV, which has esterase activity, and thogotovirus, which has a glycoprotein (GP75), that is not closely related to the influenza virus HA. However, Position-Specific Iterated BLAST (PSI-BLAST) selected to query virus proteins, revealed several viral envelope glycoproteins that did show significant similarity to the HA of RbtOV such as koala retrovirus, gibbon ape leukemia virus, and bat coronavirus. This may be due to similarities in the leucine zipper and heptad repeat features, more common to fusion proteins.

The HA sequence of RbtOV encoded by segment 6 was predicted to contain two highly hydrophobic domains. The first was a potential fusion peptide sequence at aa 284–308, FVAIAAFVIFEVLE-LAIAIGGLGIG, similar in composition to the various Class I viral fusion proteins (Cross et al., 2009). The second was a region at the terminal end of the orf (aa 441–456), WIHIALIPLTIFAIM, that was similar to a transmembrane domain. Six potential N-linked glycosylation sites were predicted for the HA of RbtOV compared to only two for the HE of ISAV (Table 3). Other orthomyxoviruses typically have from 5 to 10 sites in their HA-like glycoproteins. The hemagglutinin of RbtOV was predicted to be slightly more acidic (pI 5.1) than that of FLUCV and THOGV (pI 5.8–5.9) and quite different from the HE or HA proteins of ISAV, FLUAV, and FLUBV (Table 3).

Strains of the fish orthomyxovirus, ISAV, show large differences in virulence that have been linked to an insertion or mutation in the cleavage site of the F protein coupled with deletions in a 35-amino acid highly polymorphic region (HPR) of the 3' or stalk portion of the HE (Plarre et al., 2012; Kibenge and Kibenge 2016). Wild-type strains of ISAV are typically of low virulence, resistant to growth in cell culture, and have a full-length amino acid sequence in the HPR (termed HPRO). However, deletions in the HPR appear to occur readily during infections of farmed salmon leading to more virulent strains (HPRΔ) that spread locally among farms producing high levels of mortality (Mjaaland et al., 2002; Nylund et al., 2003).

While HPR-deleted isolates of ISAV obtained from diseased fish can be grown in cell lines, HPRO strains of ISAV are found in several species of free-ranging fish and in cultured Atlantic salmon where they replicate without producing significant mortality (Kibenge and Kibenge 2016). Because the three strains of RbtOV studied here came from healthy fish and had similar amino acid sequences of the HA, they may represent the equivalent of the ISAV wild-type strains, but which are able to grow in the CHSE-214 cell line. However, the amino acid alignment between RbtOV and ISAV in the 3' region of the sequence is quite poor and did not allow identification of a potentially similar pattern in the HA of RbtOV.

3.11. Non-structural proteins

The full length segment 7 of RbtOV was 1097 nt in length and contained two distinct orfs encoding proteins that appeared most similar to the non-structural proteins NS1 and NEP of ISAV, also encoded by the seventh smallest viral segment. The first AUG (+1 frame) at nt 43 was predicted to encode the larger NS1 protein of 306 aa having a mass of 34.3 kDa (Table 1). The second orf began with an AUG (+2 frame) at nt 581 and was predicted to encode a smaller protein of 143 aa with a mass of 16.2 kDa. This reading frame termed NS2 or NEP is likely accessed via splicing mechanisms that may include the same start methionine as the NS1 protein. Although the predicted NS1 and NEP proteins of RbtOV were of similar sizes as their ISAV orthologs, the two viruses shared low sequence identities of 21 and 25%, respectively (Fig. 3). Also, while the NEP proteins of RbtOV and ISAV had the same calculated pI of 4.4, the NS1 protein of RbtOV was predicted to be considerably more basic (pI 8.8) than that of ISAV at pI 5.9 (Table 3).

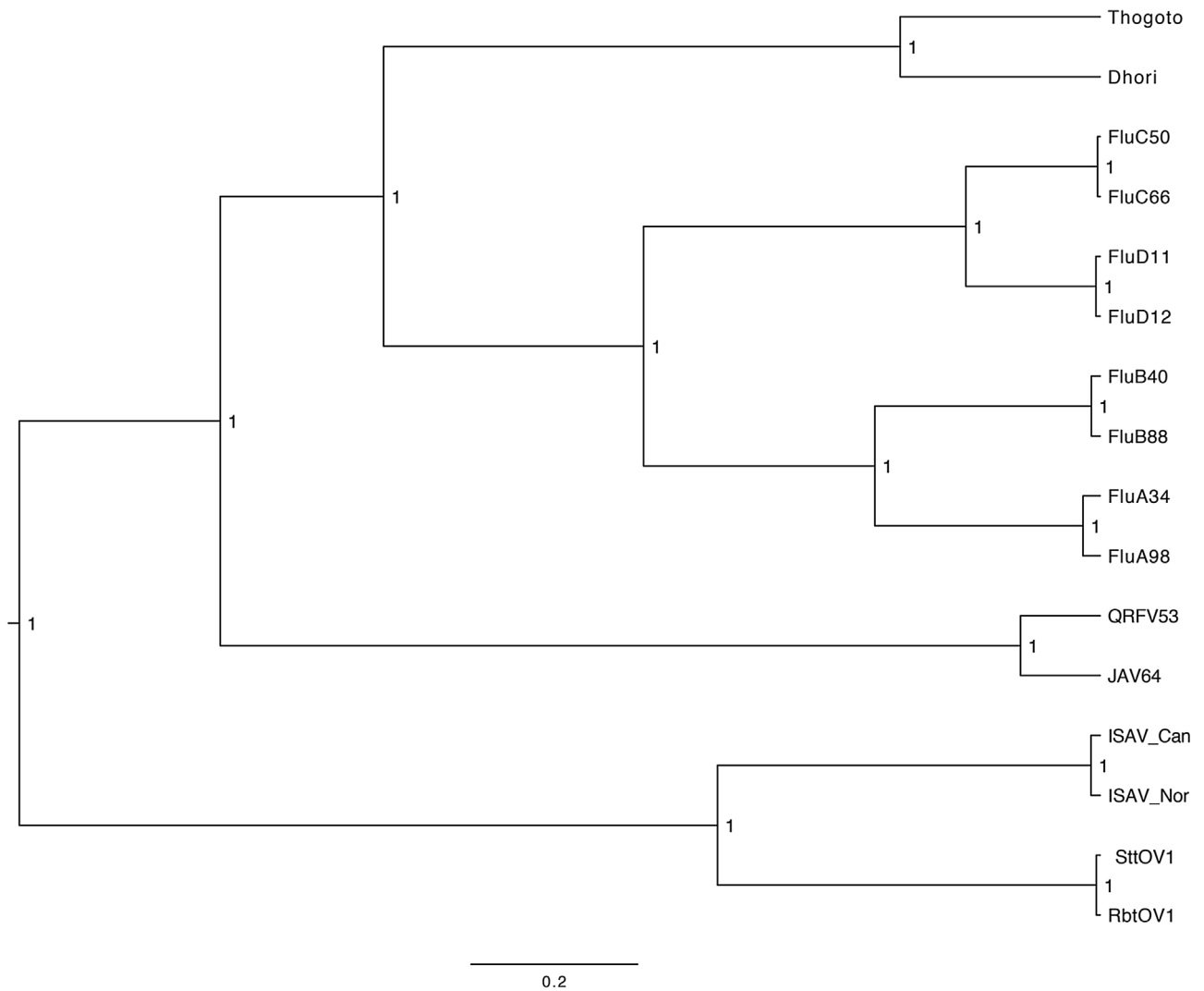


Fig. 6. Phylogenetic comparison of RbtOV and SttOV PB1 amino acid sequences with those of representative orthomyxoviruses. Two representatives of each established genus were as follows: ISAV-Glesvaer/2/1990 (ISAV_Nor; ADR77506), ISAV-Canada/CCBB (ISAV_Can; AAL67962), Influenzavirus A/PR/8/1934 H1N1 (FluA34; ABO21706), Influenzavirus A/swine/Texas/4199-2/1998 H3N2 (FluA98; AEK70339), Influenzavirus B/Lee/1940 (FluB40; AAA43767), Influenzavirus B/Yamagata/16/1988 (FluB88; ABL77264), Influenzavirus C/Ann Arbor/1/1950 (FluC50; BAD24938), Influenzavirus C/Johannesburg/1/1966 (FluC66; AAF89738), Influenzavirus D/swine/Oklahoma/1334/2011 (FluD11; AFJ19019), Influenzavirus D/bovine/France/2986/2012 (FluD12; CEE50061), Quarantfil/1953 (QRFV53; ACY56282), Johnston Atoll/1964 (JAV64; ACY56284), Thogotovirus (Thogoto; YP.145794), Dhori virus/1313/61 (Dhori; ADF56030), RbtOV-1/1997 (RbtOV1; KX882062), and SttOV-1/2014 (SttOV1; KX882070).

The NS1 protein of influenza virus A has been shown to inhibit nuclear export of mRNA and has two functional domains, an RNA-binding domain and an effector domain (Qian et al., 1994; Nemeroff et al., 1995; Wang and Krug, 1996). For the NS1 sequence of RbtOV, a poor level of similarity to influenza virus A NS1 did not allow identification of either of these domains. The nuclear export proteins of influenza viruses have been studied in depth showing the importance of a nuclear export signal or sequence (NES) between residues 12 and 21 of NS2 with characteristic hydrophobic spacing (O'Neill et al., 1998; Neumann et al., 2000; Paterson and Fodor, 2012). Whereas the NES of the influenza virus A NEP has the sequence “**ILLRMSKMQL**”, the NES of RbtOV has “**LTQMMGSLSF**” (at aa 29–38) where hydrophobic residues are shown in bold. Two additional, somewhat hydrophobic, domains (aa 83–104; aa 113–134) in the NEP sequence of RbtOV were similar to those near the C-terminus of influenza virus A, where two alpha helices C1 (aa 64–85) and C2 (aa 94–115) are located (Paterson and Fodor, 2012).

3.12. Membrane or matrix proteins

Two viral membrane or matrix proteins of RbtOV are encoded on the smallest segment 8 and were identified as putative M1 and M2 (Table 1). The first AUG (+2 frame) at nt 38 begins an orf that encodes a smaller protein of 207 aa with a predicted mass of 23.2 kDa (M1). The second AUG (+1 frame) at nt 88 begins an orf that encodes a larger protein of 235 aa with a predicted mass of 25.6 kDa (M2). Based primarily on the location of the two orfs, predicted protein sizes, and amino acid alignments, the matrix proteins of RbtOV showed the greatest similarity to their ISAV orthologs, but the percent amino acid identities between the sequences encoding these two proteins was low (20–21%) for both the M1 and M2 (Fig. 3). However, the amino acid sequences revealed no significant matches with any orthomyxoviruses when analyzed using BLASTp. Pairwise alignments of the RbtOV matrix proteins with orthologs of other representative orthomyxoviruses were also low for M1 (16–21%) and M2 (20–27%). Both the M1 and M2 proteins of RbtOV were predicted to be highly basic with a pI of 9.4. While

this appears similar to the M1 proteins of ISAV and influenzaviruses that range from pI 9.2–9.7, it was unlike the M2 proteins of ISAV and other representative influenzaviruses that are typically quite acidic with a range from pI 4.4–5.7 (Table 3). The deduced M1 amino acid sequence of RbtOV-1 contains three N-linked glycosylation sites, whereas the M2 protein only has one.

3.13. Phylogenetic and pairwise analyses

Phylogenetic analyses of aligned nucleotide and amino acid sequences of the RbtOV-1 isolate with representative orthomyxoviruses allowed separation into the anticipated genera of *Orthomyxoviridae*. Both Bayesian and maximum likelihood methods, using either the complete PB1 nucleotide sequence or the deduced amino acid sequence, produced trees with similar topologies. These analyses indicated that RbtOV and SttOV are isolates of the same virus species and most closely related to ISAV, the sole species within the genus *Isavirus* (Fig. 6). Further analyses with additional viral genes would aid in confirmation of this finding. This relationship is further supported by evidence that RbtOV has the same genome segment order as that of ISAV as well as the closest percent amino acid identities for most of the other encoded proteins. However, the overall levels of nucleotide or amino acid sequence identities between RbtOV/SttOV and ISAV shown in Fig. 3 are sufficiently low to indicate they are clearly separate virus species. Furthermore, our phylogenetic analysis and pairwise comparison of the PB1 sequences of RbtOV and SttOV with other known orthomyxoviral taxa revealed that the distances between RbtOV/SttOV and other taxa are greater than the intragenic diversity of any of the established genera within the family *Orthomyxoviridae*. Consequently, we could not assign RbtOV/SttOV into any previously described genus and the viruses potentially represent prototypes in a new genus, tentatively named *Mykissvirus* based on the species name of the original host, pending ICTV approval.

3.14. RT-PCR for fish orthomyxoviruses

The RT-PCR amplification using RNA extracted from the RbtOV-1 isolate and from strains of ISAV from Canada (CCBB) and Norway (Bremnes) performed equally well using the degenerate RT-PCR primers. The semi-nested PCR easily detected at least a 1:10,000 dilution of the original RNA extract (data not shown). All PCR amplicons were confirmed to be the proper viral strain by DNA sequencing indicating the degenerate RT-PCR assay may be useful for identifying these, and perhaps other, fish orthomyxoviruses in the future.

4. Conclusions

This study describes the isolation and characterization of a new fish orthomyxovirus, RbtOV, of rainbow and steelhead trout (*Oncorhynchus mykiss*). The complete sequence of all eight genomic RNA segments of the RbtOV-1 isolate was determined and compared with orthologs from other orthomyxoviruses to gain insights into important functional domains and features. Additionally, a partial PB1 sequence from a second isolate of RbtOV from rainbow trout (RbtOV-2) and a nearly complete genome of a third isolate from steelhead trout (SttOV-1) were compared to the initial isolate of RbtOV and to other orthomyxoviruses. These isolates appear to be members of the same virus species and sufficiently distinct from other orthomyxoviruses to merit consideration as a member of a new genus in the family *Orthomyxoviridae*. *In vitro* growth conditions were optimized, a plaque assay developed for infectious virus quantification, and a reverse transcription – polymerase chain reac-

tion (RT-PCR) assay was modified for detection and identification of similar fish orthomyxoviruses in the future.

Conflict of interests

The authors declare that no competing interests exist.

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References

- Aspehaug, V., Mikalsen, A.B., Snow, M., Biering, E., Villoing, S., 2005. Characterization of the infectious salmon anemia virus fusion protein. *J. Virol.* 79, 12544–12553.
- Bacharach, E., Mishra, N., Briese, T., Zody, M.C., Tsoref, J.E.K., Zamostiano, R., Berkowitz, A., Ng, J., Nitido, A., Corvelo, A., Toussaint, N.C., Abel Nielsen, S.C., Hornig, M., Del Pozo, J., Bloom, T., Ferguson, H., Eldar, A., Lipkin, W.I., 2016. Characterization of a novel orthomyxo-like virus causing mass die-offs of tilapia. *mBio* 7 (2), 00431–516. <http://dx.doi.org/10.1128/mbio.00431-16>.
- Bankevich, A., Nurk, S., Anstipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477.
- Batts, W.N., Winton, J.R., 1989. Enhanced detection of infectious hematopoietic necrosis virus and other fish viruses by pretreatment of cell monolayers with polyethylene glycol. *J. Aquat. Anim. Health* 1, 284–290.
- Bouchard, D., Keleher, W., Opitz, H.M., Blake, S., Edwards, K.C., Nicholson, B.L., 1999. Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. *Dis. Aquat. Org.* 35, 131–137.
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. *BMC Bioinform.* 10, 421. <http://dx.doi.org/10.1186/1471-2105-10-421>.
- Chu, C., Fan, S., Li, C., Macken, C., Kim, J.H., Hatta, M., Neumann, G., Kawaoka, Y., 2012. Functional analysis of conserved motifs in influenza virus PB1 protein. *PLoS One* 7 (5), e36113. <http://dx.doi.org/10.1371/journal.pone.0036113>.
- Cottet, L., Cortez-San Martin, M., Tello, M., Olivares, E., Rivas-Aravena, A., Vallejos, E., Sandino, A.M., Spencer, E., 2010. Bioinformatic analysis of the genome of infectious salmon anemia virus associated with outbreaks with high mortality in Chile. *J. Virol.* 84, 11916–11928.
- Cross, K.J., Langley, W.A., Russell, R.J., Skehel, J.J., Steinhauer, D.A., 2009. Composition and functions of the influenza fusion peptide. *Prot. Pept. Lett.* 16, 766–778.
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7, 214.
- Ducatez, M.F., Pelletier, C., Meyer, G., 2015. Influenza D virus in cattle, France, 2011–2014. *Emerg. Infect. Dis.*, 2011–2014 [Internet] <http://dx.doi.org/10.3201/eid2102.141449>.
- Falk, K., Namork, E., Rimstad, E., Mjaaland, S., Dannevig, B.H., 1997. Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *J. Virol.* 71, 9016–9023.
- Glenn, J.A., Emmenegger, E.J., Grady, C.A., Roon, S.R., Gregg, J.L., Conway, C.M., Winton, J.R., Hershberger, P.K., 2012. Kinetics of viral load and erythrocytic inclusion body formation in Pacific herring artificially infected with erythrocytic necrosis virus. *J. Aquat. Anim. Health* 24, 195–200.
- Hahn, C.M., Iwanowicz, L.R., Cormman, R.S., Conway, C.M., Winton, J.R., Blazer, V.S., 2015. Characterization of a novel hepadnavirus in the White Sucker (*Catostomus commersonii*) from the Great Lakes Region of the USA. *J. Virol.* 89, 11801–11811.
- Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 22, 160–174.
- Henikoff, S., Henikoff, J.G., 1992. Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. U. S. A.* 89, 10915–10919.

- International Committee on the Taxonomy of Viruses, 2005. *Orthomyxoviridae*. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Inc, Academic Press, London, pp. 681–693.
- International Committee on the Taxonomy of Viruses, 2014. Online 2014 release: <http://www.ictvonline.org/virusTaxonomy.asp>.
- Kibenge, F.S.B., Kibenge, M.J.T., 2016. *Orthomyxoviruses of fish*. In: Kibenge, F.S.B., Godoy, M.G. (Eds.), *Aquaculture Virology*. Elsevier Inc, Academic Press, London, pp. 299–326.
- Kibenge, F.S.B., Lyaku, J.R., Rainnie, D., Hammell, K.L., 2000. Growth of infectious salmon anaemia virus in CHSE-214 cells and evidence for phenotypic differences between virus strains. *J. Gen. Virol.* 81, 143–150.
- Kozak, M., 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* 266, 19867–19870.
- Krossoy, B., Hordvik, I., Nilsen, F., Nylund, A., Endresen, C., 1999. The putative polymerase sequence of infectious salmon anaemia virus suggests a new genus within the *Orthomyxoviridae*. *J. Virol.* 73, 2136–2142.
- Lamb, R.A., Kolakofsky, D., 2001. Paramyxoviridae: the viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T.P., Roizman, B., Straus, S.E. (Eds.), *Fields Virology*. 4th ed. Lippincott Williams & Wilkins, Philadelphia, pp. 577–604.
- Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10 (3), R25. <http://dx.doi.org/10.1186/gb-2009-10-3-r25>, Epub 2009 Mar 4.
- Lannan, C.N., Winton, J.R., Fryer, J.L., 1984. Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro* 20, 671–676.
- Li, C., Shi, M., Tian, J., Lin, X., Kang, Y., Chen, L., Qin, X., Xu, J., Holmes, E.C., Zhang, Y., 2015. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. *eLife* 4, <http://dx.doi.org/10.7554/eLife.05378>.
- Martin, M., 2011. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. <http://journal.embnet.org/index.php/embnetjournal/article/view/200/458>.
- Merour, E., LeBerre, M., Lamoureux, A., Bernard, J., Bremont, M., Biacchesi, S., 2011. Completion of the full-length genome sequence of the infectious salmon anaemia virus, an aquatic orthomyxovirus-like, and characterization of mAbs. *J. Gen. Virol.* 92, 528–533.
- Mjaaland, S., Rimstad, E., Falk, K., Dannevig, B.H., 1997. Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. *J. Virol.* 71, 7681–7686.
- Mjaaland, S., Hungnes, O., Teig, A., Dannevig, B.H., Thorud, K., Rimstad, E., 2002. Polymorphism in the infectious salmon anemia virus hemagglutinin gene: importance and possible implications for evolution and ecology of infectious salmon anemia disease. *Virology* 304, 379–391.
- Muhire, B.M., Varsani, A., Martin, D.P., 2014. SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS One* 9 (9), e108277. <http://dx.doi.org/10.1371/journal.pone.0108277>.
- Nemeroff, M.E., Qian, X., Krug, R.M., 1995. The influenza virus NS1 protein forms multimers *In Vitro* and *In vivo*. *Virology* 212, 422–428.
- Neumann, G., Hughes, M.T., Kawaoka, Y., 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J.* 19, 6751–6758.
- Ng, T.F.F., Zhang, W., Sachsenroder, J., Kondov, N.O., da Costa, A.C., Vega, E., Holtz, L.R., Wu, G., Wang, D., Stine, C.O., Antonio, M., Mulvaney, U.S., Muench, M.O., Deng, X., Ambert-Balay, K., Pothier, P., Vinje, J., Delwart, E., 2015. A diverse group of small circular ssDNA viral genomes in human and non-human primate stools. *Virus Evol.* 1, vev017.
- Nylund, A., Devold, M., Plarre, H., Isdal, E., Aarseth, M., 2003. Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. *Dis. Aquat. Org.* 56, 11–24.
- O'Neill, R.E., Talon, J., Palese, P., 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO J.* 17, 288–296.
- Paterson, D., Fodor, E., 2012. Emerging roles for the influenza A virus nuclear export protein (NEP). *PLoS Pathog.* 8 (12), e1003019. <http://dx.doi.org/10.1371/journal.ppat.1003019>.
- Plarre, H., Nylund, A., Karlsen, M., Brevik, O., Saether, P.A., Vike, S., 2012. Evolution of infectious salmon anaemia virus (ISA virus). *Arch. Virol.* 157, 2309–2326. <http://dx.doi.org/10.1007/s00705-012-1438-0>.
- Poch, O., Blumberg, B.M., Bougueleret, L., Tordo, N., 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J. Gen. Virol.* 71, 1153–1162.
- Presti, R.M., Zhao, G., Beatty, W.L., Mihindukulasuriya, K.A., Travassos da Rosa, A.P.A., Popov, V.L., Tesh, R.B., Virgin, H.W., Wang, D., 2009. Quarantill, Johnston Atoll, and Lake Chad viruses are novel members of the family *Orthomyxoviridae*. *J. Virol.* 83, 11599–11606.
- Qian, X., Alonso-Caplen, F., Krug, R.M., 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J. Virol.* 68, 2433–2441.
- Stadler, T., 2009. On incomplete sampling under birth-death models and connections to the sampling-based coalescent. *J. Theor. Biol.* 261, 58–66.
- Stamatakis, A., 2015. Using RAxML to infer phylogenies. *Curr. Protoc. Bioinformatics* 51 (September (6)), <http://dx.doi.org/10.1002/0471250953.bi0614s51>, 14.1–14.
- Suzuki, T., Takahashi, T., Guo, C., Hidari, K.I.J., Miyamoto, D., Goto, H., Kawaoka, Y., Suzuki, Y., 2005. Sialidase activity of influenza A virus in an endocytic pathway enhances viral replication. *J. Virol.* 79, 11705–11715.
- USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society–Fish Health Section), 2014. Standard procedures for aquatic animal health inspections. In: AFS-FHS Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens, 2014 edition.
- Wang, W., Krug, R.M., 1996. The RNA-binding and effector domains of the viral NS1 protein are conserved to different extents among influenza A and B viruses. *Virology* 223, 41–50.
- Winton, J., Batts, W., deKinkelin, P., LeBerre, M., Bremont, M., Fijan, N., 2010. Current lineages of the epithelioma papulosum cyprini (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells. *J. Fish Dis.* 33, 701–704.
- World Organization for Animal Health, 2009. 6th ed. In: *Manual of Diagnostic Tests for Aquatic Animals*. Office International des Epizooties, Paris.
- Zerbino, D.R., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* 18 (May (5)), 821–829. <http://dx.doi.org/10.1101/gr.074492.107>, Epub 2008 Mar 18.